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SURVEY AND SUMMARY

A base pair at the bottom of the anticodon stem is reciprocally preferred for discrimination of cognate tRNAs by *Escherichia coli* lysyl- and glutaminyl-tRNA synthetases

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ABSTRACT

Although the yeast amber suppressor $tRNA^{Tyr}$ is a good candidate for a carrier of unnatural amino acids into proteins, slight misacylation with lysine was found to occur in an *Escherichia coli* protein synthesis system. Although it was possible to restrain the mislysylation by genetically engineering the anticodon stem region of the amber suppressor $tRNA^{Tyr}$, the mutant tRNA showing the lowest acceptance of lysine was found to accept a trace level of glutamine instead. Moreover, the glutamine-acceptance of various $tRNA^{Tyr}$ transcripts substituted at the anticodon stem region varied in reverse proportion to the lysine-acceptance, similar to a 'seesaw'. The introduction of a C31–G39 base pair at the site was most effective for decreasing the lysine-acceptance and increasing the glutamine-acceptance. When the same substitution was introduced into *E.coli* $tRNA^{Lys}$ transcripts, the lysine-accepting activity was decreased by 100-fold and faint acceptance of glutamine was observed. These results may support the idea that there are some structural element(s) in the anticodon stem of tRNA, which are not shared by aminoacyl-tRNA synthetases that have similar recognition sites in the anticodon, such as *E.coli* lysyl- and glutaminyl-tRNA synthetases.

INTRODUCTION

Aminoacylation is a key step in the expression of genetic information, and is required for precise translation of the nucleotide sequence (genetic code) into the corresponding amino acid sequence. In general, the sites at which an aminoacyl-tRNA synthetase (aaRS) discriminates the cognate tRNA are located at a small region of the tRNA, designated the 'identity elements' of the tRNA (1). In a narrow sense, the identity elements are composed of positive and negative determinants. The positive determinants are defined by nucleotides whose mutations lead to strong losses in specific aminoacylation efficiency. Such nucleotides generally interact with the cognate aaRS by hydrogen bonding, for example, and are conserved among isoaccepter tRNAs. On the other hand, the negative determinants are thought to exist to prevent the false charging and are defined by nucleotides whose transplantations into tRNA damage cognate aminoacylation.

It is believed that an aaRS charges the cognate amino acid correctly to its cognate tRNA by precisely discriminating its own identity elements mentioned above. However, it is often the case that a tRNA with a mutation at the anticodon is recognized by several aaRSs and charged with their cognate amino acids *in vitro* (2) and even *in vivo* (3).

The amber suppressor tRNA is often adopted as a carrier of unnatural amino acids into proteins because it can be derived from virtually any tRNA by introducing appropriate mutations in the anticodon providing the CUA anticodon which can decode the UAG amber codon. In order to create an 'alloprotein' containing an unnatural amino acid at a specific site, the amber suppressor tRNA must not be charged with more than one species of amino acid in the protein synthesis system, because the produced protein must be unequivocal at the site specified by the amber codon. Recently, Wang *et al*. (4) reported the creation of an 'orthogonal' tRNA derived from *Methanococcus jannashii* $tRNA^{Tyr}$, which is not aminoacylated by any endogenous aaRSs and is specific for the amber codon. Moreover, Kowal *et al*. (5) demonstrated that an amber suppressor tRNA derived from *Escherichia coli* initiator $tRNA_f^{Met}$ functions as an orthogonal tRNA. We succeeded previously in creating such a tRNA derived from yeast $tRNA^{Tyr}$ with

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orthogonality to some extent (6). However, in a previous work, we showed that this amber suppressor $tRNA^{Tyr}$ (WT suppressor $tRNA^{Tyr}$) was slightly lysylated by *E.coli* endogenous lysyl-tRNA synthetase (LysRS), probably because the second position of the anticodon (U35) is an identity element of *E.coli* LysRS (7). In the same work, we tried to repress its lysine-acceptance in order to improve its orthogonality in an *E.coli* translation system, and have succeeded in effectively repressing the lysine-acceptance by changing several nucleotides in the WT suppressor $tRNA^{Tyr}$. The substitutions of G–C or C–G base pairs for three A–U base pairs at the anticodon stem of WT suppressor $tRNA^{Tyr}$ were most effective to repress the lysine-acceptance among lots of changes. The resulting mutant suppressor $tRNA^{Tyr}$ transcript with the lowest lysine-accepting activity (AS-GCr) appeared to function effectively as an orthogonal tRNA, at least in an *in vitro* translation system (7).

The results of the previous work mentioned above raised two questions. One is the question whether the AS-GCr mutant functions as an orthogonal tRNA also in an *in vivo* translation system. The other is which G–C base pair(s) introduced into the WT suppressor $tRNA^{Tyr}$ contribute to the repression of its lysine-acceptance.

Here, through these studies, we serendipitously found that the AS-GCr mutant accepted glutamine, albeit very slightly. We also report that the charging between glutaminylation and lysylation of a series of anticodon stem mutants from WT suppressor $tRNA^{Tyr}$ shows a clear inverse correlation. Moreover, we suggest that there may be some novel structural element(s) in the anticodon stem of *E.coli* $tRNA^{Lys}$ that are different from the identity elements in a narrow sense and are used in reciprocal manners for discrimination between *E.coli* LysRS and glutaminyl-tRNA synthetase (GlnRS).

MATERIALS AND METHODS

Materials

Aminoacyl-tRNA synthetases and transcripts of wild-type $tRNA^{Lys}$, wild-type $tRNA^{Gln}$ and *E.coli* native $tRNA^{Lys}$ were part of our laboratory stocks. *E.coli* LysRS and GlnRS were prepared as described in Ref. (8). Oligodeoxyribonucleotides and 2′-*O*-Me-modified oligonucleotides were purchased from Hokkaido System Science or Nippon Bio Service. Amino acids labeled with ^{14}C were purchased from Moravek Biochemicals.

In vivo suppression experiments

The pGEMEXAS-GCr encoding the amber suppressor $tRNA^{Tyr}$ mutant that had the lowest lysine-accepting activity (7) was digested with XbaI and HindIII, and then ligated to the XbaI and HindIII sites of pMW118 (Nippon Gene). The resulting plasmid, pMWAS-GCr, and pMWSup (6) encoding 'original' yeast amber suppressor $tRNA^{Tyr}$ were transformed into the host cells, *E.coli* CA274 (HfrC $lac_{am}125$ trp_{am}), on Luria–Bertani (LB) plates containing 50 μg/ml ampicillin. Consequently, each transformant was grown overnight in the LB media containing 50 μg/ml ampicillin and 1 mM isopropyl-β-D-thiogalactopyranoside. Additionally, *E.coli* CA274 and Q13 (having wild-type *lacZ*) containing pMW118 were grown in the same way in order to measure the β-galactosidase activity as a negative and positive control, respectively. Suppression activities were quantitatively estimated by measuring the β-galactosidase activities using the protocol of a β-galactosidase Enzyme Assay System (Promega). Briefly, cells from 1 ml culture were suspended in 100 μl lysis buffer attached in the kit and were disrupted by a sonicator (Bioruptor, Tohsho Denki, Japan). The cell extract was directly used for the β-galactosidase enzyme assay. One unit of β-galactosidase was defined as the amount of the enzyme that hydrolyzed 1 μmol of *o*-nitrophenyl-β-D-galactopyranoside to *o*-nitrophenol and galactose per minute at pH 7.5 and 37°C. Protein concentrations of the cell extract were estimated according to Ref. (9).

Plasmids, mutagenesis and preparation of tRNA transcripts

All the plasmids used for *in vitro* transcription were derived from pGEMEX-1 (Promega). The *E.coli* $tRNA^{Lys}$(T34C) gene, in which T34 of the anticodon was substituted with C, was synthesized and cloned between the XbaI and HindIII sites of pGEMEX-1 and the resulting plasmid was designated pGEMEX-Lys(T34C). Mutagenesis was performed using pGEMEX-supTyr(T4C) (10) or pGEMEX-Lys(T34C) as a template and the protocol of a QuikChange® kit (Stratagene). The obtained plasmids were as follows: pGEMEXAS-1, pGEMEXAS-3, pGEMEXAS-4, pGEMEXAS-13, pGEMEXAS-14, pGEMEXAS-34, pGEMEX-U31A39 and pGEMEX-G31C39 were used for preparing a series of amber suppressor $tRNA^{Tyr}$ transcripts; and pGEMEX-U31A39(K), pGEMEX-G31C39(K) and pGEMEX-C31G39(K) were used for preparing mutant $tRNA^{Lys}$ transcripts. For example, in order to construct pGEMEXAS-1, the following two 45mer oligodeoxynucleotides (mutation sites are underlined) were used: 5′-GTT GGT TTA AGG CGC AAG CCT CTA AAG CTT GAG ATC GGG CGT TCG-3′ and 5′-CGA ACG CCC GAT CTC AAG CTT TAG AGG CTT GCG CCT TAA ACC AAC-3′. Other plasmids were constructed using the same method with proper oligodeoxynucleotides. Each mutation was confirmed by dideoxy sequencing methods according to the manufacturer's protocol (Amersham). Each tRNA transcript was prepared according to a previously described method (10), except for the use of a suitable plasmid and a 2′-*O*-Me-modified primer, 5′-T(Gm)G TGG GTC GTG CAG GAT T-3′, for $tRNA^{Lys}$ transcripts. All the tRNA transcripts used in this study are shown in Figure 1.

Aminoacylation assay

The time course of aminoacylation was measured at 37°C for lysylation and glutaminylation. The reaction mixture (total volume, 50 μl) contained 100 mM Tris–HCl (pH 7.6), 10 mM $MgCl_2$, 40 mM KCl, 4 mM ATP, ~10 μM [^{14}C]amino acid, 0.25 A_{260} units of tRNA or transcript and 12.5 μg of LysRS or 25 μg of GlnRS from *E.coli*. The aminoacylation activities of the tRNA transcripts were estimated by the acceptance of [^{14}C]amino acid per one A_{260} unit of tRNA transcript.

Lysylation reactions to determine the kinetic constants for each tRNA transcript were carried out at 37°C in 100 μl reaction mixtures as described in Ref. (11) with a

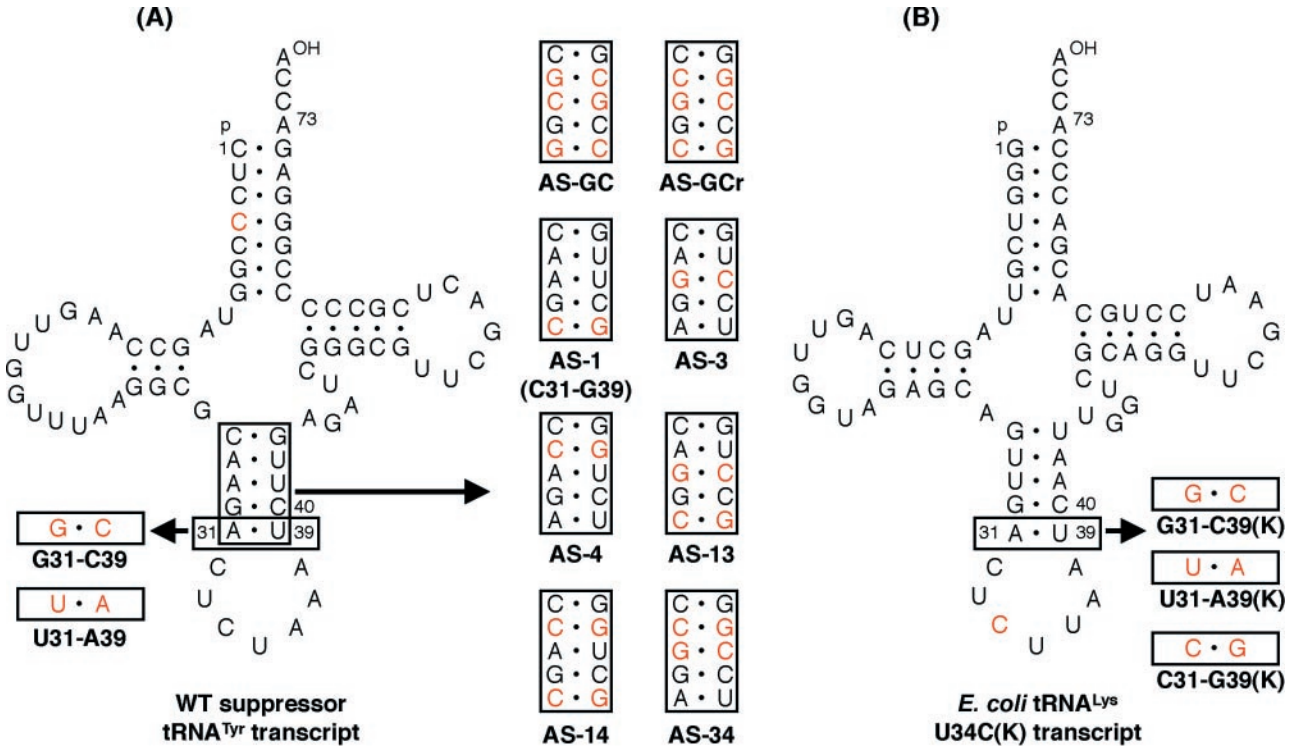

Figure 1. Nucleotide sequences and designated names of the tRNA transcripts used in this study. Boxed areas show mutated regions of the tRNAs and red characters indicate mutated nucleotides. (**A**) Yeast suppressor $tRNA^{Tyr}$ transcripts and (**B**) *E.coli* $tRNA^{Lys}$ transcripts.

slight modification. The initial rates of aminoacylation were determined by using different concentrations of the tRNA transcript at fixed concentrations of lysine (16 μM) and ATP (4 mM) and suitable concentrations of *E.coli* LysRS, which gave reasonable kinetic plots for determining the apparent kinetic constants.

Since the glutamine-acceptances of the tRNA transcripts derived from *E.coli* $tRNA^{Lys}$ transcript were very low, k_{cat}/K_m values were determined according to the technique of Schulman and Pelka (12). Briefly, the reaction mixture (total volume, 10 μl) contained 100 mM Tris–HCl (pH 7.6), 10 mM $MgCl_2$, 40 mM KCl, 4 mM ATP, 30 μM [^{14}C]glutamine, 8 μM *E.coli* GlnRS and suitable concentrations of tRNA transcripts (8, 16 or 32 μM). The initial velocities of glutamine-accepting activity were calculated from the acceptance of [^{14}C]glutamine after 10 min.

RESULTS

In vivo suppression experiment with a suppressor tRNA mutant that had the lowest lysine-acceptance *in vitro*

In the previous work, we showed that the yeast amber suppressor $tRNA^{Tyr}$ was slightly mischarged with lysine in an *E.coli* translation system (7). However, we have also succeeded in effectively repressing the lysine-accepting activity by changing all the base pairs of the anticodon stem in this tRNA into G–C base pairs (7). Our first interest in this study was to clarify whether or not the 'optimized' suppressor $tRNA^{Tyr}$ (AS-GCr; Figure 1) that had the lowest lysine-acceptance shows the orthogonality in an *E.coli* *in vivo* translation system. Therefore, we investigated the suppression efficiency with *E.coli* CA274 that has an amber mutation in the β-galactosidase gene. It is known that the β-galactosidase activity is restored when this amber codon is suppressed by any of the following amino acids: Ser, Gln, Tyr, Lys, Leu or Trp (13). Table 1 shows the *in vivo* suppression efficiencies when plasmids encoding the yeast amber suppressor $tRNA^{Tyr}$ (pMWSup) and the AS-GCr (pMWAS-GCr) were transformed into *E.coli* CA274. Under the previously reported conditions, *E.coli* transformed with pMWSup alone grew as white colonies on X-Gal plate (6), indicating that yeast amber suppressor $tRNA^{Tyr}$ did not function efficiently in *E.coli* cells in the absence of yeast tyrosyl-tRNA synthetase (TyrRS). In fact, the β-galactosidase activity of *E.coli* CA274 harboring pMWSup was greatly lower than that of Q13 having the wild-type enzyme (Table 1, rows 1 and 3). However, it was much higher than that of negative control (Table 1, rows 2 and 3). This result is in agreement with the indication by Wang *et al.* (14) that *E.coli* CA274 overexpressing yeast amber suppressor $tRNA^{Tyr}$ formed blue colonies on X-Gal plate and a previous result that it was slightly mischarged with lysine (7). In the case of the AS-GCr, the *in vivo* suppression efficiency was decreased, as expected (Table 1, rows 3 and 4). Moreover, the reduction rate of the *in vivo* suppression efficiency in this study was similar to that of the *in vitro* suppression efficiency studied previously using an *E.coli* cell-free translation system (7). However, β-galactosidase activity of *E.coli* CA274 harboring pMWAS-GCr was still higher than that of the negative control (Table 1, rows 2 and 4). Therefore, we suspected that the AS-GCr was also mischarged with another amino acid other than lysine.

Table 1. *In vivo* suppression efficiency with the plasmids encoding yeast amber suppressor tRNAs^{Tyr}

E.coli + plasmid	*lacZ*	Yeast amber suppressor tRNA^{Tyr}	β-Galactosidase activity (U/mg protein)[a]	Suppression efficiency[b] (%)
Q13 + pMW118	Wild type	—	21.9	—
CA274 + pMW118	Amber	—	0.005	0.02
CA274 + pMWSup	Amber	Wild type[c]	0.065	0.30
CA274 + pMWAS-GCr	Amber	AS-GCr	0.024	0.11

[a]One unit of β-galactosidase was defined as the amount of the enzyme that hydrolyzed 1 μmol of *o*-nitrophenyl-β-D-galactopyranoside to *o*-nitrophenol and galactose per minute at pH 7.5 and 37°C.
[b]The suppression efficiencies were estimated by the percentage of β-galactosidase activities from *E.coli* CA274 against the activity of the wild-type enzyme from *E.coli* Q13.
[c]The wild-type amber suppressor tRNA^{Tyr} gene used in this experiment possesses an original thymidine at the fourth nucleotide instead of the cytidine shown in Figure 1 [see Refs (7,10)].

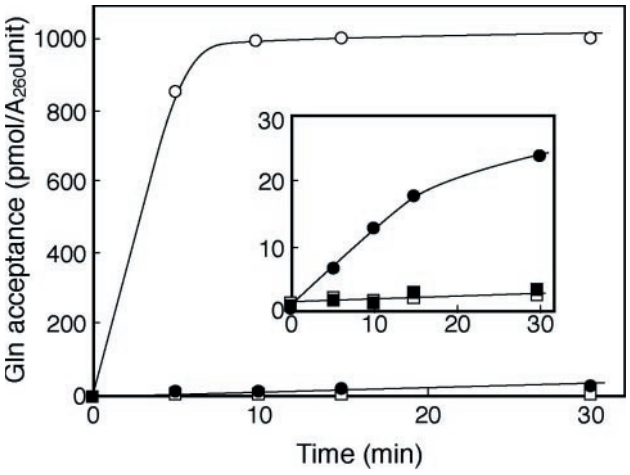

Figure 2. Time course of glutaminylation of the mutant AS-GCr, which shows the lowest lysine-acceptance. Open and closed circles show the glutamine-acceptance of the $tRNA_2^{Gln}$ transcript and the AS-GCr mutant, respectively. Closed squares show the glutaminylation of WT suppressor tRNA^{Tyr} and open squares indicate the negative control without tRNA. The inset shows a graph in which the vertical axis is magnified in order to observe the faint acceptance of glutamine.

The amber suppressor tRNA showing the lowest lysine-acceptance is glutaminylated by GlnRS

The misacylation of the AS-GCr mutant was measured by using concentrated GlnRS, glutamyl-tRNA synthetase (GluRS) and proryl-tRNA synthetase (ProRS) (1 mg/ml each), since these aaRSs have similar identity elements at the anticodon (GlnRS and GluRS) or in the accepter stem (ProRS) (1). The enzyme concentration used is ~100-fold higher than our normally used concentration (7). GluRS and ProRS did not charge any amino acids at all (data not shown), whereas GlnRS charged glutamine to the AS-GCr (Figure 2), albeit very slightly. The glutamine-acceptance of the WT suppressor tRNA^{Tyr} was not detectable, indicating that the slight suppression of β-galactosidase by the AS-GCr (Table 1) may reflect its misaminoacylation with glutamine.

Inverse correlation between lysine- and glutamine-acceptance

The fact that the AS-GCr mutant was mischarged with glutamine motivated us to investigate which G–C base pairs introduced into the anticodon stem of yeast amber suppressor tRNA^{Tyr} effectively influenced its lysine- and glutamine-acceptance. We then prepared a series of anticodon stem mutants that covered all the combinations of the G–C base pairs introduced into the AS-GCr (Figure 1).

Table 2. Lysine and glutamine-acceptances of a series of suppressor tRNAs^{Tyr} mutated in the anticodon stem

Suppressor tRNA	Lysine-acceptance (pmol/A_{260} unit)	Glutamine-acceptance (pmol/A_{260} unit)
WT suppressor tRNA^{Tyr}	85	4
AS-GC	7	14
AS-GCr	4	22
AS-1	8	18
AS-3	61	5
AS-4	69	7
AS-13	4	20
AS-14	5	22
AS-34	68	6

Table 2 and Figure 3 show the lysine- or glutamine-accepting activities of a series of anticodon mutants. The aminoacylation level was estimated by the acceptance of lysine or glutamine per one A_{260} unit of tRNA transcript. Interestingly, the mutant having lower lysine-acceptance had higher glutamine-acceptance and vice versa. On the whole, a good inverse correlation between lysine- and glutamine-acceptance was observed (Figure 3), suggesting that repression of the lysine-accepting activity enhances the glutamine-accepting activity, similar to a 'seesaw' effect.

Changing the base pair 31–39 of suppressor tRNA^{Tyr} influences its aminoacylation

Substitution of the base pair A31–U39 to C31–G39 was found to be the most effective for repressing the lysine-acceptance, although these mutations concomitantly aroused a slight glutamine-accepting activity (compare AS-3, AS-4 and AS-34 with AS-1, AS-13 and AS-14, respectively in Figure 3). In order to study the effect of the 31–39 mutations in detail, the lysine- or glutamine-accepting activities of WT suppressor tRNA^{Tyr} (A31–U39) or its mutants G31–C39, U31–A39 and AS-1 (C31–G39) were compared (Figure 4). Although introducing the U31–A39 base pair induced slightly more effective repression of the lysine-acceptance than introduction of the G31–C39 base pair, both these mutations showed moderate repression

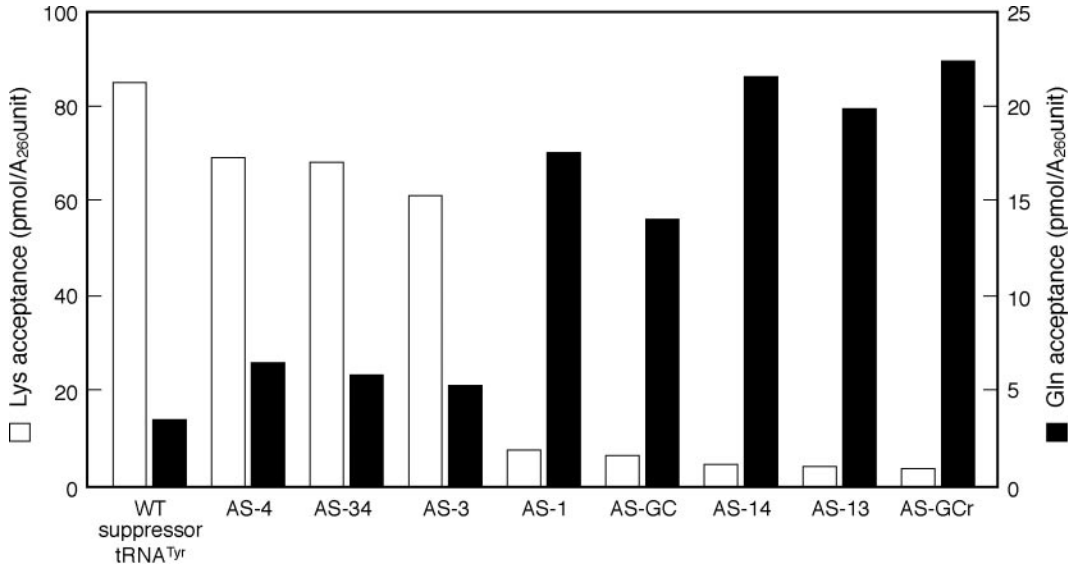

Figure 3. Lysine- and glutamine-accepting activities of a series of anticodon stem mutants derived from yeast suppressor $tRNA^{Tyr}$. Aminoacylation activities were estimated by the plateau level of aminoacylation. Open and closed bars show the lysine- and glutamine-acceptance, respectively. The tRNAs are arranged according to their lysine-acceptance starting from the left.

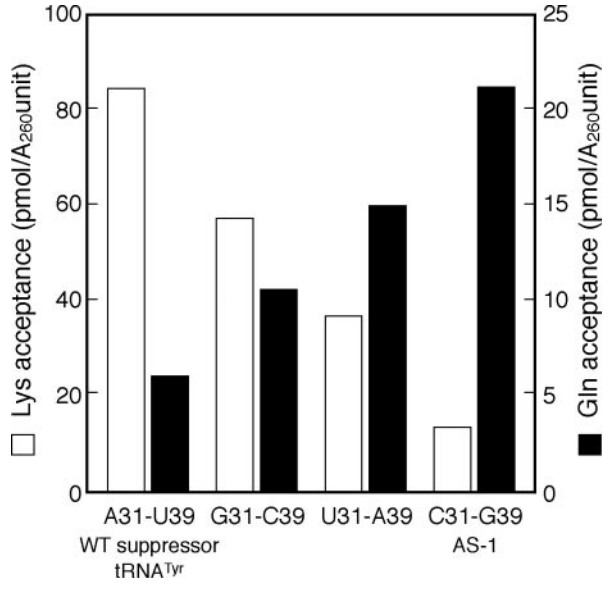

Figure 4. Correlation between the type of the base pair 31–39 in the suppressor $tRNA^{Tyr}$ and lysine- or glutamine-acceptance. Open and closed bars show the lysine- and glutamine-acceptance, respectively. The tRNAs are arranged according to their lysine-acceptance starting from the left.

of the lysine-accepting activity. Interestingly, a seesaw correlation between lysine- and glutamine-acceptance was also observed in this case (Figure 4). These results indicate that there may be some structural element(s) at the base pair 31–39 that are reciprocally preferred for discrimination of tRNA between LysRS and GlnRS.

Mutations at the base pair 31–39 of *E.coli* $tRNA^{Lys}$ diminish its lysine-acceptance

We prepared mutants of *E.coli* $tRNA^{Lys}$ with substitutions at the base pair 31–39, in order to examine whether the above-mentioned structural element(s) at this base pair were also found in the *E.coli* lysine and glutamine systems. However, there is a crucial problem associated with studies using *E.coli* $tRNA^{Lys}$ transcripts, since it has already been reported that modification (5-methylaminomethyl-2-thiouridine; mnm^5s^2U) of the first nucleotide of the anticodon of $tRNA^{Lys}$ is important for the lysine-accepting activity [(15) and Table 3, rows 1 and 2). On the other hand, since many bacteria have another $tRNA^{Lys}$ species with the anticodon CUU ($tRNA^{Lys}$(CUU)), their LysRS should be able to recognize the $tRNA^{Lys}$(CUU) effectively. Therefore, we tried to introduce C34 into an *E.coli* $tRNA^{Lys}$ transcript (designated the U34C(K) transcript) and then measured its lysine-accepting activity. As shown in Table 3, its lysine-accepting activity increased by ~30-fold compared to the wild-type (K) transcript. In light of this result, various base pairs (U–A, G–C and C–G) were introduced at the base pair 31–39 of the U34C(K) transcript. Table 3 shows the lysine-accepting activities of this series of *E.coli* $tRNA^{Lys}$ transcripts. The order of the decrease in the lysine-accepting activity (k_{cat}/K_m) was G–C [G31–C39(K)], U–A [U31–A39(K)] and C–G [C31–G39(K)], which is highly consistent with the order shown in Figure 4. Interestingly, none of the K_m values changed very much. Since Rould *et al.* (16) commented that the proper conformational change of GlnRS induced by $tRNA^{Gln}$ is probably reflected in the k_{cat} values of the kinetic parameters, this result may indicate that mutants at the base pair 31–39 could be equally bound by LysRS, but that after binding, the conformational changes of $tRNA^{Lys}$ and/or LysRS necessary to express the catalytic activity must somehow be hindered.

Only introduction of C31–G39 into *E.coli* $tRNA^{Lys}$ arouses glutamine-acceptance

Next, it was examined whether or not a series of $tRNA^{Lys}$ transcripts could accept glutamine. Only the C31–G39(K)

Table 3. Kinetic parameters for the lysine-accepting activities of *E.coli* $tRNA^{Lys}$ and its derivatives

$tRNA^{Lys}$	(Anticodon)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})	Relative activity (fold value)[a]
Native (K)	(mnm^5s^2UUU)	1.35	2.50	1850	87.3
Wild-type (K) transcript	(UUU)	4.30	0.0029	0.67	0.032
U34C (K)	(CUU)	1.37	0.029	21.2	1
U31–A39(K)	(CUU)	2.28	0.0020	0.88	0.042
G31–C39(K)	(CUU)	2.15	0.0047	2.2	0.10
C31–G39(K)	(CUU)	2.58	0.00069	0.27	0.013

[a]The relative aminoacylation efficiencies were calculated using the k_{cat}/K_m value for the U34C(K) transcript as a reference.

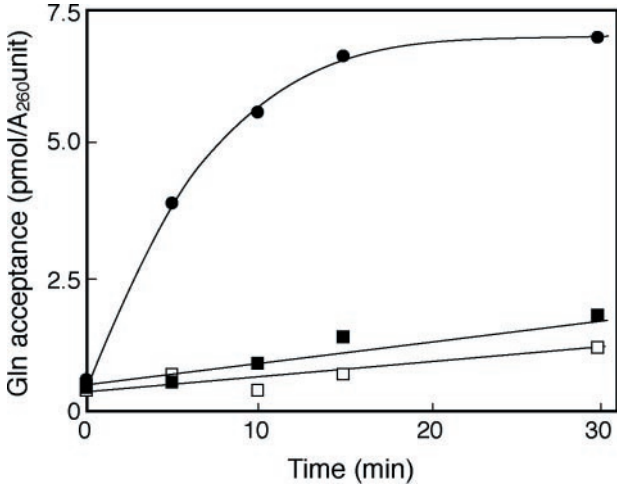

Figure 5. Time course of glutaminylation of the C31–G39 mutant derived from the *E.coli* $tRNA^{Lys}$(U34C) transcript. Open and closed squares show the glutamine-acceptance of the wild-type $tRNA^{Lys}$ transcript and U34C(K) mutant, respectively. Closed circles show the glutaminylation of the C31–G39(K) mutant from *E.coli* $tRNA^{Lys}$(U34C).

mutant accepted glutamine, albeit very faintly (Figure 5), whereas the other mutants did not accept glutamine (data not shown). In order to assess the glutamine-accepting activities of the mutants quantitatively, their kinetic parameters were tried to measure. Since the glutamine-acceptances of the tRNA transcripts derived from *E.coli* $tRNA^{Lys}$ transcript were very low, k_{cat}/K_m values were determined according to the technique of Schulman and Pelka (12). Briefly, when the tRNA concentration is far below the K_m, the slope of the linear plot of initial velocity of the aminoacylation versus tRNA concentration gives V_{max}/K_m. Even with this protocol, only the k_{cat}/K_m value of the C31–G39(K) was measurable and its activity was $\sim 10^5$-fold lower than that of wild-type $tRNA^{Gln}$ transcript (Table 4). Nevertheless, the fact that C31–G39(K) accepted glutamine was an unexpected result because C31–G39 is not conserved among $tRNA^{Gln}$ isoacceptors. In this context, the C31–G39 base pair is a positive determinant for the glutamine system, but different from the identity element of $tRNA^{Gln}$ in a narrow sense.

DISCUSSION

We have shown that the amber suppressor $tRNA^{Tyr}$ derived from yeast and its mutants are misaminoacylated by LysRS and/or GlnRS, albeit very slightly. More than 10 years ago, Normanly *et al*. (17) set out to construct a complete collection

Table 4. The glutamine-accepting activities of *E.coli* $tRNA^{Lys}$ derivatives

tRNA transcript	(anticodon)	k_{cat}/K_m (mM^{-1} s^{-1})	Relative activity (fold value)[a]
Wild-type (Q) transcript	(CUG)	48	1
U34C (K)	(CUU)	$<10^{-4}$	ND
U31–A39(K)	(CUU)	$<10^{-4}$	ND
G31–C39(K)	(CUU)	$<10^{-4}$	ND
C31–G39(K)	(CUU)	4.3×10^{-4}	9.0×10^{-6}

[a]The relative aminoacylation efficiencies were calculated using the k_{cat}/K_m value for the wild-type $tRNA^{Gln}$ transcript [wild-type (Q) transcript] as a reference; ND, not determined.

of *E.coli* amber suppressor tRNAs corresponding to each of the 20 amino acids. They showed that the suppressor tRNAs thus constructed could be classified into three groups based on the results of *in vivo* suppression of dihydrofolate reductase and subsequent protein sequencing. The class I suppressors inserted the predicted amino acids, whereas the class II or III suppressors predominantly inserted glutamine or lysine, respectively. In this context, mischarging of our yeast suppressor $tRNAs^{Tyr}$ with lysine and/or glutamine is not a surprising result. However, the most unexpected results of the present study are that the mutations which repressed the lysine-acceptance enhanced the glutamine-acceptance and that the charging level showed a good inverse correlation between lysine- and glutamine-acceptance, similar to a seesaw. In addition, the balance of the lysine- and glutamine-acceptance was considerably affected by mutations of the base pair 31–39 at the bottom of the anticodon stem. Although the identity elements in a narrow sense have been already reported to mnm^5s^2U34, U35, U36 and A73 for lysine (15,17,18) and to U1–A72, G2–C71, G3–C70, G10, pyrimidine34, U35, G36, A37, U38 and G73 for glutamine system (19–22), it has not been investigated whether the base pair 31–39 represents the identity element of lysine and glutamine systems. In addition, A31–U39 (Ψ39 is almost always the case) is a common feature of usual tRNAs and not specific to lysine or glutamine systems. Moreover, no significant interaction between A31–Ψ39 of *E.coli* $tRNA^{Lys}$ and *Thermus thermophilus* LysRS was reported in the crystal structure of the complex (23). Although the crystal structure of the *E.coli* $tRNA^{Gln}$ and GlnRS complex has also been reported, no interaction between A31–Ψ39 and GlnRS was mentioned (16). Therefore, it seems that the base pair 31–39 cannot be an identity element for both systems.

When the same mutation at the base pair 31–39 in a series of yeast amber suppressor $tRNA^{Tyr}$ was introduced into the *E.coli* $tRNA^{Lys}$ transcript, the lysine-acceptance of the

$tRNA^{Lys}$ mutants was considerably reduced. Notably, substitution of A31–U39 of $tRNA^{Lys}$ to C31–G39 resulted in a reduction of its lysine-accepting activity by ~100-fold, in accordance with the result that the same substitution introduced into WT suppressor $tRNA^{Tyr}$ effectively repressed the misacylation with lysine. Interestingly, the same mutation aroused glutamine-acceptance in each case.

We found a similar example in the report by Normanly *et al*. (17). They showed that the amber suppressors originating from $tRNA_1^{Ile}$and $tRNA_2^{Ile}$ charged glutamine and lysine, respectively. Interestingly, the base pair 31–39 of $tRNA_1^{Ile}$ is A31–Ψ39 and that of $tRNA_2^{Ile}$ is C31–G39. In terms of their *in vivo* experiments, the inverse correlation between lysine- and glutamine-acceptance resembling a seesaw would have been explained. Since LysRS and GlnRS strongly recognize the middle base of the anticodon (U35), both aaRSs could compete for one tRNA *in vivo*. In this case, the lower affinity for the tRNA of one aaRS should be a relative advantage for the other aaRS. However, since our measurements of lysine- or glutamine-acceptance were carried out separately *in vitro*, the balance of the affinity between LysRS and GlnRS should have been out of consideration. Consequently, we hypothesize that some kind of structural element(s) may exist in the anticodon stem, and that LysRS and GlnRS prefer the element(s) reciprocally.

These element(s) are clearly different from the identity elements in a narrow sense, because C31–G39 was a positive determinant for the glutamine system, but is not conserved among $tRNA^{Gln}$ isoacceptors. These element(s) also seem to be different from the 'non-permissive elements' found in yeast $tRNA^{Phe}$ by Frugier *et al*. (24). These non-permissive elements are sequences outside the identity elements of tRNA, and only a specific combination of mutations has an antideterminant effect on the amino acid acceptance that is compensated by a second-site mutation. However, all the mutants of the base pair 31–39 decreased the lysine-accepting activity, similar to the classical identity element. Thus, we propose that the structural element(s) preferred by LysRS and GlnRS may be the flexibility for a conformational change of the anticodon stem. Since the base pair 31–39 is located at the edge of the anticodon stem, it seems plausible that this base pair strongly affects the flexibility of the anticodon stem. This hypothesis can explain our results that substitution to G31–C39 or U31–A39 moderately repressed the lysine-acceptance. The former can be explained by the fact that the hydrogen bond of G–C is stronger than that of A–U, while the latter can be explained by the strength of the base stacking. When the second base in the duplex is a pyrimidine (C40 in our case; see Figure 1), the first purine (A39) has a stronger base stacking in the A-formed duplex than U39, since the overlapping area of a purine is larger than that of a pyrimidine (25). Although the case of A-formed DNA duplexes is described in this reference, the stability of RNA duplexes should be similar. In fact, the melting temperature of an RNA duplex composed of ACCGGU (52.4°C) is higher than that of UCCGGA (50.1°C), even though the numbers of A–U/G–C pairs are the same (26).

We consider that the flexibility of the anticodon stem is closely related to the overall conformational change in tRNA. After LysRS or GlnRS initially recognizes the major identity element U35, the tRNA would be fitted to the aaRS. It is probable that LysRS may loosen the anticodon stem of its cognate tRNA, whereas GlnRS may tighten it. The crystal structures of each aaRS/tRNA complex (16,23) support this idea. In the LysRS/$tRNA^{Lys}$ complex, the electron density of $tRNA^{Lys}$ was unclear and could not be observed, except for the anticodon region. Cusack *et al*. (23) mentioned that a pre-productive complex was formed in which the anticodon was docked correctly under their crystallization conditions, but the rest of the tRNA was not fully engaged with LysRS. On the other hand, in the GlnRS/$tRNA^{Gln}$ complex, the electron density of tRNA was clearly observed and GlnRS elongated the anticodon stem of $tRNA^{Gln}$ by two non-Watson–Crick-type base pairs (32–38 and 33–37) after tRNA binding (16). Moreover, very recently, our group solved the crystal structure of the yeast TyrRS/$tRNA^{Tyr}$ complex (M. Tsunoda *et al*., manuscript in preparation). In this structure, the electron density of the anticodon stem was not observable. Probably, the base pair(s) of the anticodon stem of yeast $tRNA^{Tyr}$ may be unstable in the complex. It is possible that this flexibility in the anticodon stem causes mischarging of lysine to the yeast WT suppressor $tRNA^{Tyr}$.

With regard to the biological meaning of the difference in flexibility of the anticodon stem, our hypothesis is as follows. Since LysRS and GlnRS recognize similar identity elements, there is a possibility that both aaRSs compete for one tRNA. Therefore, both enzymes have to discriminate their cognate tRNAs by the flexibility of the anticodon stem during the fitting process, and this may be the reason why the flexibility of the anticodon stem is evolutionarily conserved. Nevertheless, further structural investigations are required in the future, for example, using the NMR technique, in order to fully elucidate the stability of the anticodon stem and its effect on the flexibility at the base pair 31–39.

Although we have tried to find similar examples that the flexibility of tRNA influences the recognition by the cognate aaRS, we could find very few reports directly discussing such an issue. Recently, Guigou and Mirande (27) reported the necessity of the flexibility of tRNA to the effective activation of mammalian arginyl-tRNA synthetase. The phenomenon that the G–U base pairs found in the stem structure of $tRNA^{Arg}$ influences its k_{cat} value of the arginine activation is very similar to our results. Probably, there may be many other fine-tuning mechanisms in which the flexibility of tRNA participates, as aaRSs effectively meet the cognate tRNAs.

Finally, through this investigation, we feel that it is very difficult to design a tRNA that is not mischarged at all by a rational combination of point mutations since some unknown and reciprocal fine-tuning mechanisms like this case would be revealed one after another. In this connection, the 'orthogonal' suppressor tRNA created by Wang *et al*. (4) was selected from a random pool of tRNA mutants by an evolutionary way. The molecular evolutionary approach would probably be more effective way for preparing such a tRNA.

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